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Citation for published version:

Rahaman, YA, Yeap, SK, Tan, SW, Hair-Bejo, M, Fakurazi, S, Kaiser, P & Omar, AR 2015, 'In vitro characterization of chicken bone marrow-derived dendritic cells following infection with very virulent infectious bursal disease virus', *Avian Pathology*, vol. 44, no. 6, pp. 452-462.
<https://doi.org/10.1080/03079457.2015.1084997>

Digital Object Identifier (DOI):

[10.1080/03079457.2015.1084997](https://doi.org/10.1080/03079457.2015.1084997)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Avian Pathology

Publisher Rights Statement:

This is an Accepted Manuscript of an article published by Taylor & Francis in Avian Pathology on 25th August 2015, available online: <http://www.tandfonline.com/doi/abs/10.1080/03079457.2015.1084997>

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Publisher: Taylor & Francis & Houghton Trust Ltd

Journal: *Avian Pathology*

DOI: 10.1080/03079457.2015.1084997

Cavp-2015-0061.R1

In vitro characterization of chicken bone marrow-derived dendritic cells following infection with very virulent infectious bursal disease virus

Y.A. Rahaman^{1,2}, S.K. Yeap¹, S.W. Tan¹, M. Hair-Bejo^{1,2}, S. Fakurazi¹, P. Kaiser³ & A.R. Omar^{1,2*}

¹*Institute of Bioscience, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia*

²*Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul*

Ehsan ³*The Roslin Institute and R (D)SVS, University of Edinburgh, Easter Bush, Midlothian EH25 9RG, UK*

Short title: Dendritic cells infected with vvIBDV

** To whom correspondence should be addressed. Tel: +60 3-8947 2111, Fax: +60 3-8947 2101, E-mail: aro@upm.edu.my*

Received: 5 June 2015

Abstract

Infectious bursal disease (IBD) is caused by infectious bursal disease virus (IBDV), an immunosuppressive virus that targets immune cells such as B cells and macrophage. However, the involvement of dendritic cells (DCs) during IBDV infection is not well understood. In this study the *in vitro* effects of live and inactivated very virulent IBDV (vvIBDV) UPM0081 on bone marrow derived DCs (BM-DC) were characterized and compared with BM-DC treated with lipopolysaccharide (LPS). Morphologically, BM-DC treated with LPS and vvIBDV showed stellate shape when compared to immature BM-DC. In addition, LPS-treated and both live and inactivated vvIBDV-infected BM-DC expressed high levels of double positive CD86 and MHC class II antigens (>20%). vvIBDV-infected BM-DC showed significantly higher numbers of apoptotic cells compared to LPS. Replication of vvIBDV was detected in the infected BM-DC as evidenced by the increased expression of VP3 and VP4 IBDV antigens based on flow cytometry, real-time PCR and immunofluorescence tests. Levels of different immune-related genes such as IL-1 β , CXCLi2 (IL-8), IL-18, IFN- γ , IL-12 α , CCR7 and TLR3 were measured after LPS and vvIBDV treatments. However, marked differences were noticed in the onset and intensity of the gene expression between these two treatment groups. LPS was far more potent than live and inactivated vvIBDV in inducing the expression of IL-1 β , IL-18 and CCR7 while expression of Th1-like cytokines, IFN- γ and IL-12 α were significantly increased in the live vvIBDV treatment group. Meanwhile, the expression of TLR3 was increased in live vvIBDV-infected BM-DC as compared to control. Inactivated vvIBDV-treated BM-DC failed to stimulate IFN- γ , IL-12 α and TLR3 expressions. This study suggested that BM-DC may serve as another target cells during IBDV infection which require further confirmation via *in vivo* studies.

Key words: Bone Marrow Dendritic Cell (BM-DC), Infectious Bursal Disease (IBD), Infectious Bursal Disease Virus (IBDV), Lipopolysaccharide (LPS), Immune-related genes, Apoptosis

Introduction

Infectious bursal disease (IBD) is an extremely contagious immunosuppressive disease of young chicks, caused by infectious bursal disease virus (IBDV) (Vervelde & Davison, 1997; van den Berg, 2000). IBDV is a bi-segmented dsRNA virus belonging to the genus *Avibirnavirus* of the family *Birnaviridae* (Dobos *et al.*, 1995). The IBDV genome is about 6 kb in total and is comprised of two segments, A and B. There are two serotypes, serotype 1 and 2; although only serotype 1 is capable of producing disease in chickens and studies have shown that pheasants, partridges, quails, and guinea fowls are resistant to classical serotype 1 IBDV infection (McFerran *et al.*, 1980; Ismail *et al.*, 1988).

In general, serotype 1 can be classified into various subtypes/strains based on virus virulence. Classical, variant and very virulent strains are capable of breaking through maternal antibody and cause lesions in the bursa. Variant strains can break maternal antibody to a greater extent than the classical strain, resulting in mortality of less than 5%; whereas classical strains induce more severe bursal lesions compared to the variant strains (Rauf *et al.*, 2011). However, very virulent strains generally cause severe and acute pathological effects, as well as higher mortality, compared to the classical strains (Williams & Davison, 2005). In addition, typical very virulent strains may cause up to 100% mortality in specific-pathogen-free (SPF) chickens (van den Berg *et al.*, 2004). Meanwhile, mild and vaccine strains do not

cause mortality or clinical symptoms, but bursal lesions are dependent on the virulence of the virus (Rautenschlein *et al.*, 2005).

The virus replication occurs in lymphoid cells, especially the bursa of Fabricius of young chickens, specifically targeting actively dividing IgM-bearing B cells (Withers *et al.*, 2006). Hence, the humoral immune response is compromised due to depletion of B cells, and subsequently the chickens develop immunosuppression and secondary bacterial infection. IgM-bearing B cells are not the only target cells for IBDV as macrophage and other immune cells also serve as the target of IBDV infection, which functionally carry the virus to the bursa and other tissues following IBDV infection (Khatri *et al.*, 2005).

Correspondingly, dendritic cells (DCs) serve as antigen-presenting cells (APCs) and are categorized as unique professional APCs. They play a central role in the induction of primary immune responses and the generation of immunological memory. DCs are present in peripheral tissues and have the ability to uptake, process and present antigen to prime T cell responses (Steinman, 2007). DCs function as sentinels of the immune system, and as such are found in almost all parts of body, including mucosal surfaces, skin epidermis, interstitial tissues, peripheral blood, as well as lymphoid and non-lymphoid tissue (Liu, 2001). In the event of exposure to an infectious agent, DCs recognise the antigen via various pattern-recognition receptors (PRRs) such Toll-like receptors (TLRs) and cytoplasmic NOD-like receptors to initiate an innate immune response (Mogensen, 2009). Stimulated DCs produce several types of cytokines and undergo activation and maturation by expression of high levels of MHCII on their surface, together with the expression of co-stimulatory molecules, for instance CD83, CD80, CD40 and CD86, accompanied by morphological changes, particularly by extended cytoplasmic process (Banchereau & Steinman, 1998).

In chicken, DCs progenitor from bone marrow; follicular DCs from secondary lymphoid organs namely spleen, Harderian glands, Payer's patches and cecal tonsils; as well

as Langerhans cells have been isolated and characterized (Gallego *et al.*, 1995a, b; Igyarto *et al.*, 2006, 2007; del Cacho *et al.*, 1993, 2008; Wu *et al.*, 2010). The involvements of chicken DCs during viral infection and immune responses are not well characterized. However, recent studies have characterised the functions of BM-DC in response to avian influenza and vaccine strain of IBDV (Vervelde *et al.*, 2013; Liang *et al.*, 2015).

BM-DCs have been characterized by enrichment with granulocyte monocyte colony stimulating factor (GM-CSF) and Interleukin-4 (IL-4) (Wu *et al.*, 2010). Lipopolysaccharide (LPS) is recognized as one of the activator of DCs maturation in mammals (Bai *et al.*, 2002) and BM-DCs in chickens (Wu *et al.*, 2010). LPS is a major constituent of the cell wall of most gram negative bacteria, and is able to enhance the immune response to soluble antigens. In the present study, we hypothesize that IBDV can induce BM-DCs maturation similar to LPS. Therefore, the aims of this study are to study the involvement of BM-DCs during *in vitro* vvIBDV infection and to characterize the differences between LPS-treated and vvIBDV-infected BM-DCs.

Materials and Methods

Chickens. Specific-pathogen-free (SPF) embryonated eggs were purchased from Veterinary Research Institute (VRI), Ipoh, Perak. The eggs were hatched and transferred to the Animal House Facility, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia. The chicks were fed *ad libitum* and given water twice a day. The chickens were killed at 3 to 5 weeks old for harvesting the bone marrow. All procedures were approved by Animal Care and Use Committee, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM/IACUC/AUP-R051/2014).

***In vitro* generation of chicken BM-DCs.** BM-DCs obtained from femurs were cultured as described by Wu *et al.* (2010) with modifications. Briefly, BM-DC at 3×10^6 cells were seeded in 6 well plate and 9×10^6 cells in T-25 tissue culture flasks and cultured in pre-warmed RPMI-1640 media (Sigma-Aldrich, Singapore) supplemented with 10% chicken serum (Gibco, USA), 1 U/ml penicillin and 1 μ g/ml streptomycin (Gibco, USA) and 1% L-Glutamine (Gibco, USA), and enriched with recombinant chicken IL-4 and GM-CSF for 6 days under 5% CO₂ at 37°C. BM-DC were infected on day 6 post-culture.

Infection with IBDV. Very virulent (vv) IBDV strain UPM0081 was first isolated in a Northern state of Peninsular Malaysia during an IBD outbreak in 2000. The isolate has been characterized as a vvIBDV strain based on the VP2 sequence (accession no. AY520910) and a pathogenicity study (Tan *et al.*, 2004). Enriched BM-DCs were prepared in three groups, with each group in triplicate, mock-infected BM-DCs as control, BM-DCs treated with 200 ng/ml LPS from *Salmonella enterica serotype enteritidis* and BM-DCs infected with 0.5 MOI live and inactivated (incubated at 60°C for 30 minutes) vvIBDV UPM0081. For vvIBDV, the cells were infected for two hours and the extracellular virus was washed. BM-DCs were harvested at 3, 6, 12 and 24 hours following LPS treatment or vvIBDV infection for subsequent assays.

Morphological examination of BM-DCs. The morphology of BM-DCs was assessed using an inverted microscope (Olympus, USA) and a scanning electron microscope (SEM) (JOEL, Japan) followed method as described by Naqi & Millar (1979). Prior to studying BM-DC morphology with SEM, the cells were grown on Poly-L-Lysine round coverslips in a 6-well plate and fixed in 4% glutaraldehyde, washed with 0.1 M sodium cacodylate buffer and fixed

again with 1% osmium tetroxide. Then the cells were dehydrated in acetone and dried using carbon dioxide to critical point. After mounting, the specimens were viewed using JOEL SEM (USA) with the setting of 15KV.

Immunophenotyping of BM-DC using flow cytometry. Phenotype of BM-DC was assessed using immunofluorescence-labelling with mouse anti-chicken monoclonal antibody (mAb) against major histocompatibility complex (MHC) class II (Southern Biotech, USA) and CD86 (Southern Biotech, USA). Before use the antibodies were conjugated with different fluorophores with the lightning-link conjugation system following the manufacturer's instructions (Innova Bioscience, UK), where peridinin chlorophyll A protein (PerCp) and allophycocyanin (APC) were used for MHCII and CD86, respectively. 1×10^6 cells were incubated with the antibody in the dark, at 4°C, for 45 minutes and washed twice. The percentages (mean \pm SD) of cells stained for MHCII and CD86 were determined by flow cytometry using a FACSCalibur with Cell Quest Pro software (BD Bioscience, USA).

Apoptosis study using flow cytometry. Flow cytometry was used to assess the percentage of live, apoptotic and dead BM-DC. The cells were prepared using a FITC Annexin V Apoptosis Detection Kit 1 (BD Bioscience, USA). Briefly, 1×10^6 cells were re-suspended in 1x binding buffer and incubated with FITC Annexin V and propidium iodide (PI) for 15 minutes at room temperature (RT), in the dark, before analyzing the cells with a FACSCalibur flow cytometer (BD Bioscience, USA).

Detection of IBDV using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from BM-DC using an RNeasy Mini kit (Qiagen, Germany) with DNase treatment to eliminate any DNA contamination while RNA purity and

concentration were measured using Bio-Spectrophotometer (Eppendorf, USA). cDNA was synthesized from 1 µg of RNA using iScript cDNA synthesis kit (Bio-Rad, USA).

Quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad, USA) in a 20 µl reaction with primers (100 nm) specific for the IBDV VP4 gene (Kong *et al.*, 2009). The PCR was performed on a CFX96™ Real Time System (BioRad, USA) with the following protocol: 95 °C for 5 minutes, 46 cycles of 95 °C for 10 seconds, annealing at 58.7 °C for 30 seconds, extension 72 °C for 5 seconds and melt curve analysis was performed at 70 °C to 95 °C with increments of 0.5 °C every 5 seconds/step. A standard curve was generated by using vvIBDV UPM0081 ($10^{7.4}$ EID50/ml) in order to determine the sample viral load.

Detection of IBDV antigens. Expression of IBDV antigens on BM-DCs was detected based on surface antigen staining using flow cytometry and immunofluorescence antibody test (IFAT). In both assays, mAb against IBDV VP3 (IBDV9) (Abcam, UK) conjugated with fluorescein isothiocyanate (FITC) (Innova Bioscience, UK) was incubated simultaneously with CD86 mAb conjugated with APC as described in previous section and analyzed with FACSCalibur flow cytometer. For IFAT, the cells were firstly fixed with neutral buffered 4 % paraformaldehyde (Sigma-Aldrich, Singapore) for 15 minutes at RT and permeabilized with 0.2 % Triton X-100/PBS solution for 2 minutes at RT. The sample was then blocked with 1 % BSA/PBS for 1 hour at RT and stained with 500x diluted IBDV9 mAb and CD86 mAb for 1 hour at RT in the dark. After a washing step, the cells were incubated with DAPI (Sigma-Aldrich, Singapore) and mounted on slides for observing under a confocal microscope (Olympus, USA).

Gene expression study using RT-qPCR. A gene expression study was performed using Custom TaqMan® Gene Expression Assays (Applied Biosystem, USA) by using different

primers and probe designed according to previously published sequences with modification by Kaiser *et al.* (2000); Kogut *et al.* (2003) and Wu *et al.* (2011b) that detect pro-inflammatory cytokines and chemokines such as IL-1 β , CXCLi2 and IL-18; Th1-like cytokines such as IFN- γ and IL-12 α ; DCs maturation marker CCR7 (Table 1). Meanwhile, Made-to-Order TaqMan® Gene Expression Assays (the sequence of the probe and primers were as designed by Applied Biosystem) were used for the detection of Toll-like receptor 3 (TLR3) (Assay ID: Gg03359006) while GAPDH (Assay ID: Gg03346990_g1) and β -actin (Assay ID: Gg03815934_s1) were used as housekeeping genes and qRT-PCR data were normalized against both GAPDH and β -actin. The total volume of the reaction was 20 μ l which consisted of 10 μ l TaqMan® Fast Advance Master Mix (2x), 1 μ l Custom/ Made-to-Order TaqMan® Gene Expression Assays (20x), 2 μ l cDNA template and 7 μ l nuclease-free water. The cycling program consisted of an initial activation (denaturation) step at 95°C for 5 minutes, 39 cycles of denaturation at 95°C for 3 seconds, annealing at 60°C for 30 seconds, and elongation for 20 seconds at 72°C, followed by a plate read. Mean quantification cycle values (Cq) were determined based on triplicates. Standard curves for each gene were prepared using positive control RNA extracted from HD11 cells (an avian macrophage cell line) treated with LPS. The reactions were run on a CFX96™ Real Time System (BioRad, USA).

Statistical Analysis. All quantitative data are expressed as mean \pm standard deviation. Differences between means were calculated using SPSS version 21 by using one-way ANOVA followed by Duncan post-hoc test. Statistical significance was calculated by comparison between groups at $P < 0.05$.

Results

Activation and maturation profiles of chicken BM-DCs following LPS and vvIBDV

treatment. Inverted microscope examination showed obvious differences between morphology of control and infected BM-DCs at 12 hours post-infection (hpi) (Figure 1A). Control BM-DCs were rounded in shape indicating that they were in an immature state, whilst treatment of BM-DC with LPS and vvIBDV triggered BM-DC to undergo maturation as observed by the formation of dendrites. However, the formation of dendrites in vvIBDV-infected BM-DC was not as obvious as LPS-treated cells. SEM examination of BM-DC further confirmed that vvIBDV activates BM-DC maturation as evidenced by the morphological differences (Figure 1B).

Phenotypic analysis of BM-DCs, based on surface expression of class II MHC and CD86 detected by flow cytometry, was used to assess the activation of BM-DCs (Figure 3). The gated population was >90% of all events, where a significant increase ($P<0.05$) in the surface expression of double positive MHCII and CD86 were detected in LPS-stimulated, live vvIBDV and inactivated vvIBDV-infected BM-DCs ($\geq 20\%$), as compared to the control in which only 1.8% of the cells were positive for these two markers. Nonetheless, the expression of MHCII on BM-DCs remains high before and after stimulation of LPS and vvIBDV. In fact, BM-DC treated with LPS expressed the highest percentage of both surface markers at 3 hpi (32.3%). In contrast, live and inactivated vvIBDV-infected BM-DC reached the maximum expression of 27.7 % at 24 hpi and 28.79% at 12 hpi, respectively (Table 2).

Figure 2 shows the IFAT of BM-DC stained with anti-CD86 for surface protein, DAPI staining for the nucleus, and IBDV VP3 for virus. The nuclear staining showed the BM-DC treated with LPS or vvIBDV grow in aggregates and formed dendrites. Unlike control BM-DCs, LPS and vvIBDV treated cells showed distinct and dense CD86-positive staining, as

indicated by the red colour. In addition, expression of VP3 in BM-DCs signifies the presence of IBDV, and both red and green colours reveal that the BM-DCs had undergone maturation upon vvIBDV infection.

Replication of vvIBDV in BM-DCs. Flow cytometry analysis showed *in vitro* treatment of BM-DC with LPS and vvIBDV cause a significantly decreased population of live cells over time, while in uninfected BM-DCs the live cell population is maintained at more than 90% (Figure 4). Most of the LPS-treated and vvIBDV-infected BM-DCs undergo apoptosis when compared to the control. However, there were significantly more early apoptosis cells at 3 and 12 hpi with LPS, whilst vvIBDV-induced apoptosis was more prominent at 24 hpi ($P < 0.05$). Indeed, vvIBDV significantly induced obvious late apoptosis even when compared to LPS at 12 and 24 hpi. Meanwhile, very few stimulated BM-DC with LPS and vvIBDV ($\leq 1\%$) underwent necrosis.

Evidence of vvIBDV replication in BM-DC was detected by RT-qPCR as early as 3 hpi and peaked at 12 hpi (Table 3). In addition to RT-qPCR, IBDV antigens were also detected by IFAT and flow cytometry. Using IFAT, vvIBDV replication, based on intracellular-VP3 staining, was detected in BM-DCs (Figure 2). Flow cytometric analysis of the surface expression of vvIBDV VP3 protein, was broadly in agreement with the qRT-PCR viral quantification, with the expression of this vvIBDV antigen on the surface of BM-DCs increasing from 3 hpi to 24 hpi (Table 3).

Expression levels of immune-related genes of BM-DCs following *in vitro* LPS and vvIBDV stimulation. In order to understand the role of BM-DCs in IBDV infection, several immune-related genes that are known to be involved in IBDV infection were analyzed. mRNA expression of pro-inflammatory cytokines such as IL-1 β , CXCLi2 (IL-8) and IL-18;

Th1-like cytokines such as IFN- γ and IL-12 α , DC maturation marker CCR7 and TLR3 were measured using RT-qPCR (Figure 5). In general, both LPS and live vvIBDV-treated BM-DC showed increased expression of all the immune-related genes measured, however, obvious differences were detected in the onset and intensity of cytokine expression between these two groups. LPS was far more potent than vvIBDV in inducing the expression of the pro-inflammatory cytokines IL-1 β and IL-18 in BM-DC. The expression of these cytokines was detected as early as 3 h post-treatment with LPS, however, their expression declined over time. No clear time-dependent expression of these cytokines was detected from live vvIBDV-infected cells but live vvIBDV-infected BM-DC expressed higher levels of CXCLi2 at both 3 and 6 hpi when compared to LPS treatment. On contrary, inactivated vvIBDV-infected BM-DC produces mild expression of CXCLi2 and down regulation of IL-18 and IL-1 β genes.

The expression of the Th1-like cytokines IFN- γ and IL-12 α was significantly increased at 6 hpi, with a peak expression detected at 12 hpi in vvIBDV-infected BM-DCs, whilst, LPS-treated BM-DCs expressed peak levels as early as 3 hpi and thereafter expression of IFN- γ and IL-12 α decreased. Interestingly, inactivated vvIBDV-infected BM-DC failed to induce these cytokines. In addition, the expression of CCR7, one of DCs activation marker was detected in both LPS and vvIBDV-treated BM-DC. However, the highest expression was detected at 3 hpi after LPS treatment, whilst the expression of CCR7 in live and inactivated vvIBDV-infected BM-DC was detected at 12 hpi and 24 hpi, respectively. On the other hand, the expression of TLR3 was up-regulated in live vvIBDV-infected BM-DC indicating the possible involvement of dsRNA molecules of IBDV with TLR3 in the infected BM-DCs. On contrary, TLR3 was not expressed on BM-DCs upon inactivated vvIBDV infection.

Discussion

Dendritic cells (DCs) are so-called unique APCs due to their ability to regulate the immune system, by undergoing maturation and differentiation mediated by various pattern-recognition receptors (PRRs) and subsequently expressing various surface molecules and producing a variety of cytokines following activation (Liu, 2001). Studies have shown the presence of DCs in various lymphoid tissues of chickens. However, the involvements of DCs during infection as well as in the activation of immune responses against infectious agents are not well characterized in the chicken.

IBDV is a lymphotropic virus targeting B cells (Withers *et al.*, 2006) and macrophages (Khatri *et al.*, 2005). Traditionally, B cells, macrophages and DCs are specialized white blood cells characterized as APCs that express MHCII on their surface and subsequently activate T cells upon exposure to pathogen. Hence, we hypothesized that BM-DCs are involved in IBDV infection. In this study, chicken BM-DCs were cultured in the presence of GM-CSF and IL-4 for 6 days, followed by LPS treatment or vvIBDV infection. Morphologically, vvIBDV-infected BM-DCs phenotypically resembled LPS-treated BM-DCs, however, the stellate formation of BM-DCs in the LPS-treated group was more prominent than in the vvIBDV-treated DCs (Figure 1). This finding is in agreement with previous studies on the effects of LPS on chicken BM-DC (Wu *et al.*, 2010) and murine BM-DCs (Morelli *et al.*, 2001).

Depletion of B lymphocytes in the bursa of chickens infected with vIBDV has been associated with the induction of apoptosis as well as necrosis of the infected cells (Nieper *et al.*, 1999; Yao & Vakharia, 2001). Moreover, both classical and vvIBDV were shown to induce apoptosis in an avian macrophage cell line, NCSU, at 72 hpi (Khatri & Sharma, 2007), and we have also recently shown that HD11 cells infected with vvIBDV strain UPM0081 undergo apoptosis after 24 hpi (Rasoli *et al.*, 2015). In this study, we detected higher numbers of apoptotic cells in vvIBDV-infected BM-DCs compared to LPS-treated BM-DC at 12 and

24 hpi. However, no significant level of necrosis was detected from the infected DCs within 24 hpi, although we made a similar finding as in a previous study by Wu *et al.* (2010), that the viability of the DCs drops significantly if the cultures are maintained for more than 7 days (data not shown). The role of human and murine DCs in viral infections has been well-studied and it was shown that human and murine myeloid- and monocyte-derived DCs undergo apoptosis following infection with viruses such as measles virus, foot and mouth disease virus and type A influenza virus, which may be associated with the induction of immunosuppression (Kushwah & Hu, 2010; Wu *et al.*, 2011a).

In general, immature chicken BM-DCs produce high level of MHCII and putative CD11c, moderate expression of CD40 as well as low expression of CD86. Upon maturation with LPS, the surface expression of CD86 and CD40 was significantly higher while the expression of MHCII remains high (Wu *et al.*, 2010; Ling *et al.*, 2015). In this study, double-colour expression of CD86 and MHCII were used in which MHCII served as reference molecule in order to characterize the population of BM-DCs post-LPS, live and inactivated vvIBDV treatment. In comparison to unstimulated BM-DCs which produce low population of double positive MHCII and CD86, BM-DCs stimulated with LPS, live and inactivated vvIBDV showed enhanced expression of double positive MHCII and CD86 cells. In our study, the expression was more prominent in LPS-stimulated BM-DCs as early as 3 hpi ($p < 0.05$). Inactivated vvIBDV-stimulated BM-DCs produce relatively lower expression of double positive MHCII and CD86 than live vvIBDV-infected BM-DCs at 3 hpi, 6 hpi and 24 hpi ($p < 0.05$). This finding was in contrast with Ling *et al.*, (2015) what might be attributed to different strains of IBDV studied. Nevertheless, both study demonstrated that both live and inactivated vvIBDV are capable of activating BM-DCs maturation but probably via different mechanisms, where inactivated vvIBDV viral particle or surface protein is recognized as extracellular pathogen and processed via MHC class II, while live vvIBDV dsRNA is

recognized as intracellular pathogen which activated by DCs via MHC class I antigen processing.

A study of highly pathogenic (H7N1) and low pathogenic (H5N2) avian influenza subtypes used to infect chicken BM-DCs showed that both virus subtypes were detected as early as 4 hpi (Vervelde *et al.*, 2013). Similarly, in our study, early detection of IBDV antigen by flow cytometry, IFAT and real-time PCR indicated that IBDV was replicating inside BM-DCs. The surface expression of the VP3 antigen and viral RNA (based on the VP4 gene) were detected as early as 3 hpi and the peak of infection was at 12 hpi. Previous studies have shown the presence of IBDV antigen in KUL01⁺ bursal-derived macrophages (Khatri *et al.*, 2005), whilst IBDV antigen is readily detected by IFAT in the chicken B cell line, DT40, following infection (Terasaki *et al.*, 2008). These findings might suggest that vvIBDV is able to replicate in BM-DCs and induce their maturation indicating that recognition of vvIBDV by BM-DCs do occur and that DCs are susceptible to IBDV infection. Previous studies have shown that different viruses are able to replicate in DCs, however, their replication is not always associated with the maturation of DCs. For example, viruses such as dengue virus, type A influenza virus and herpes simplex virus are able to replicate inside DCs, but impede the maturation process of the cells (Freer & Matteucci, 2009).

In highly pathogenic avian influenza H7N1 infection, chicken BM-DC were believed to cause a cytokine storm, which explained the severe immune system shutdown (Vervelde *et al.*, 2013). In this study, elevated immune-related genes such as IFN- γ , IL-12 α , IL-1 β , CXCLi2, IL-18, CCR7 and TLR3 were observed in general after LPS treatment and vvIBDV infection (Figure 5). These cytokines are believed to play an important role in IBDV infection. Additionally these cytokines were also released by human or murine DCs to promote activation of T cells to differentiate into Th-1 phenotypes. The expression of Th1-like cytokines, which include IFN- γ and IL-12 α , were significantly enhanced in vvIBDV-infected

BM-DC compared to those treated with LPS, suggesting that these cells are strongly involved in priming CMI via cytotoxic T cells activation and promote inflammation. However, BM-DC infected with inactivated vvIBDV failed to stimulate the expression of IFN- γ and IL-12 α , which might be due to the nature of extracellular pathogen that promote Th2-like cytokines expression. Rauw *et al.* (2007) showed that chicken IFN- γ was highly elevated in the peripheral blood during the early phase of IBDV infection due to infiltration of T cells. In addition, IL-12 is innately produced by mouse BM-DC and is involved in the differentiation of T cells into Th1 cells and stimulates expression of IFN- γ (Hsieh *et al.*, 1993).

On the other hand, it was found that LPS was far more potent than vvIBDV in stimulating the expression of pro-inflammatory cytokines such as IL-1 β and IL-18 from BM-DC, in both the time and magnitude of the response (Figure 5). This might be due to the effect of rapid activation of DCs by Toll-like receptor 4 (TLR4) and integrin PRRs molecules upon LPS stimulation (Ling *et al.*, 2014). In our study, it seems that BM-DCs infected with inactivated vvIBDV showed poor stimulation and down-regulation of these pro-inflammatory cytokines as compared to live vvIBDV-infected BM-DCs which induce strong Th-1 like cytokines and pro-inflammatory cytokines. Probably the inactivated vvIBDV-infected BM-DC is involved in inducing cytokines and associated with MHC class II processing pathway for the activation of Th-2 and B cells which require further study to proof the mechanism. In addition, the expression of cytokines such as IL-1 β , CXCLi2 (IL-8), IL-18 and IL-12 α from BM-DC infected with vvIBDV were also detected in previous studies from blood monocytes, and splenic and bursal macrophages of IBDV infected chickens (Ingrao *et al.*, 2013).

The high levels of CCR7 expression in vvIBDV infected BM-DCs indicate that the cells are undergoing maturation. Similar results have also been reported for LPS treated BM-DCs in this study and in a previous study by Wu *et al.* (2011b). Meanwhile, the up-regulation of TLR3 was only detected in live vvIBDV-infected BM-DC which suggests that the

activation is associated with recognition of TLR3 signaling pathway of the viral dsRNA. The involvement of TLR3 during virus infection has been reported for different RNA viruses such as infectious bronchitis virus (Kint *et al.*, 2015) and avian influenza virus (Karpala *et al.*, 2008; Vervelde *et al.*, 2013) and during IBDV infection of chicken embryo fibroblast cells (Wong *et al.*, 2007). Hence, IBDV is probably targeting different antigen presenting cells such as B cells, macrophage and DCs during infection.

In conclusion, both live and inactivated vvIBDV induce BM-DCs maturation, however, inactivated vvIBDV failed to induce expressions of Th1-like cytokines. In contrast, live vvIBDV is capable of replicating inside BM-DCs, induce apoptosis and stimulate strong Th-1 like cytokines. This study demonstrated the susceptibility of chicken BM-DCs towards IBDV infection but further studies are needed to characterize the role of chicken DCs during IBDV infection and vaccination.

Acknowledgements

The study was supported by an Institute of Bioscience, Higher Institution Centre of Excellence under grant (IBS HiCoE 6369101) from the Ministry of Higher Education, Government of Malaysia. Pete Kaiser is supported by a Biotechnology and Biological Sciences Research Council Institute Strategic Programme Grant to The Roslin Institute. The author was funded by Ministry of Higher Education under SLAB/SLAI programme. The author would like to thank Dr Lisa Rothwell from The Roslin Institute for proof-reading the manuscript.

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Table 1. *Quantitative Real-Time PCR Probes and Primers*

Target		Probe or Primer Sequence (5'–3')	Accession No.
IL-1β	Probe	(FAM) ^a -CACACTGCAGCTGGA-(NFQ) ^b	AJ245728
	F ^c	GCTCTACATGTCGTGTGTGATGAG	
	R ^d	TGTCGATGTCCCGCATGA	
CXCLi2	Probe	(FAM)-CTTTACCAGCGTCCTACCT-(NFQ)	AJ009800
	F	GCCCTCCTCCTGGTTTCAG	
	R	TGGCACCGCAGCTCATT	
IL-12α	Probe	(FAM)-GTCCTCTGCTTCTGCAC-(NFQ)	AY262751
	F	TGGCCGCTGCAAACG	
	R	ACCTCTTCAAGGGTGCACCTCA	
IL-18	Probe	(FAM)-CCTTCAGCAGGGATG-(NFQ)	AJ276026
	F	AGGTGAAATCTGGCAGTGGAAT	
	R	ACCTGGACGCTGAATGCAA	
IFN-γ	Probe	(FAM)-TGGCCAAGCTCCCGATGAACGA-	Y07922
	F	(NFQ)	
	R	GTGAAGAAGGTGAAAGATATCATGGA GCTTTGCGCTGGATTCTCA	
CCR7	Probe	(FAM)-	HQ269806
	F	TGAGGGTCACCATCGCTTTCAGCC-(NFQ)	
	R	CATGGACGGCGGTAAACAG TCATAGTCGTCGTGTACGTTGT	

^a FAM, 6-carboxyfluorescein; ^b NFQ, 3' nonfluorescent quencher. ^c F, forward primer; ^d R, reverse primer.

Table 2. *Percentage of CD 86 and MHC II double positive cells following LPS treatment and vvIBDV infection at different time points*

Time (hpi)	Proportion of CD 86/ MHC II double positive cells (Mean \pm SD) (%) ^A		
	LPS	Live vvIBDV	Inactivated vvIBDV
3	32.31 \pm 1.93 ^B	21.17 \pm 1.60 ^C	15.46 \pm 0.68 ^D
6	27.10 \pm 0.41 ^B	21.91 \pm 0.97 ^C	19.15 \pm 0.31 ^D
12	27.64 \pm 0.70 ^B	26.19 \pm 0.66 ^B	27.79 \pm 0.48 ^B
24	21.71 \pm 0.94 ^C	27.66 \pm 1.77 ^B	22.22 \pm 0.51 ^C

^A For control DCs the percentage of double positive cells is 1.8 \pm 0.03. The data shown are representative of three independent experiments. *^{ABCD} Values with different superscripts differ significantly at P < 0.05.

Table 3: Detection of IBDV antigens in vvIBDV infected BM-DCs

Time (hpi)	IBDV expression of DC (%) ^{a,b}	VP3 of CD86+ BM- surface	IBDV VP4 copy number (Log ₁₀) ^{a,b}
3	3.02 ± 0.13 ^A		11.91 ± 0.16 ^A
6	5.45 ± 0.29 ^A		12.94 ± 0.23 ^A
12	5.97 ± 0.47 ^B		13.11 ± 0.25 ^B
24	7.91 ± 0.87 ^C		13.07 ± 0.32 ^B

^a Surface expression of VP3 and CD86 antigens on CD86+BM-DCs following vvIBDV infection were detected using flow cytometry, whilst the expression of IBDV VP4 copy number was quantified using RT-qPCR.

^b For uninfected DCs and LPS treated DCs, there were no IBDV detected. ^{*ABC} Values with different superscripts differ significantly at $P < 0.05$. The data shown are representative of three independent experiments.

Figure Legends

Figure 1. Morphology of BM-DCs viewed using A) inverted microscope at magnification of x600; and B) SEM a) Control DCs, b) LPS-treated BM-DCs and c) vvIBDV-infected BM-DCs.

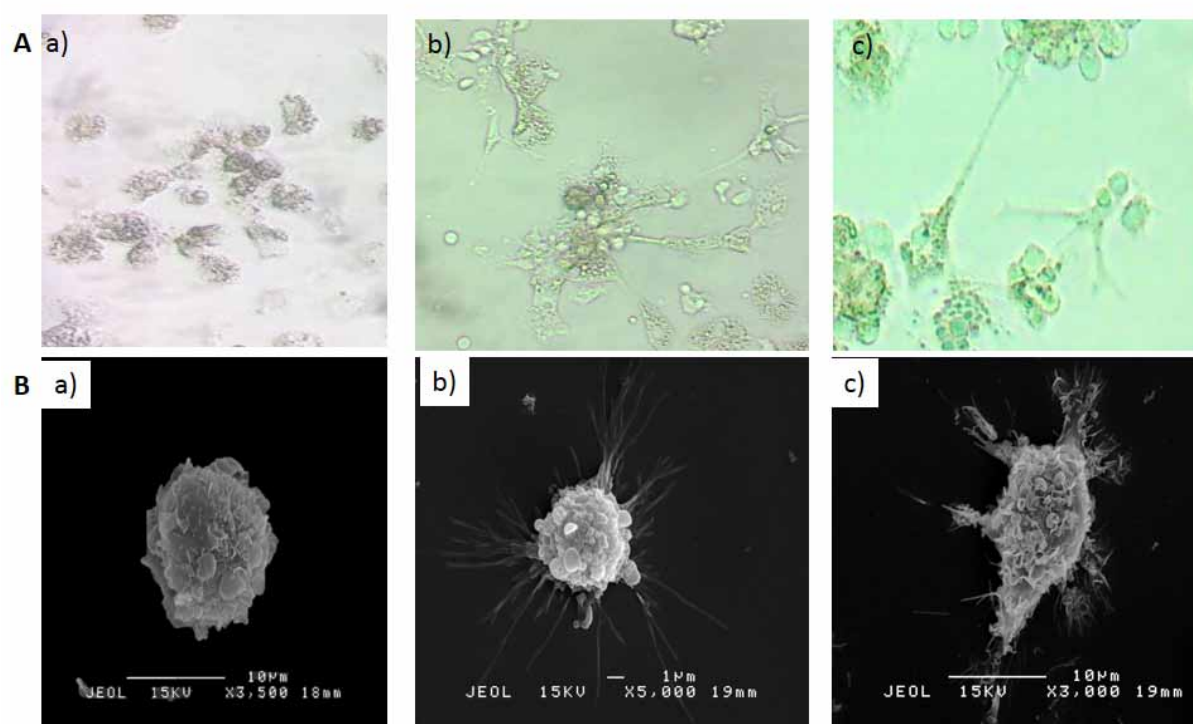
Figure 2. Immunofluorescence detection of IBDV VP3 protein in BM-DCs. Note: a) Control BM-DCs b) BM-DCs treated with LPS and c) BM-DCs infected with vvIBDV UPM0081. DAPI (blue colour) stained the nucleus, FITC-IBDV9 (green colour) stained the IBDV VP3 protein, and APC-CD86 (red colour) stained the DCs surface protein.

Figure 3. Flow Cytometric Dotplot Graphs: Percentage of CD 86 and MHC II double positive cells. Note: a) Control BM-DCs; b) LPS-treated BM-DCs; c) live vvIBDV-infected BM-DCs; and d) inactivated vvIBDV-infected BM-DCs, at different time points.

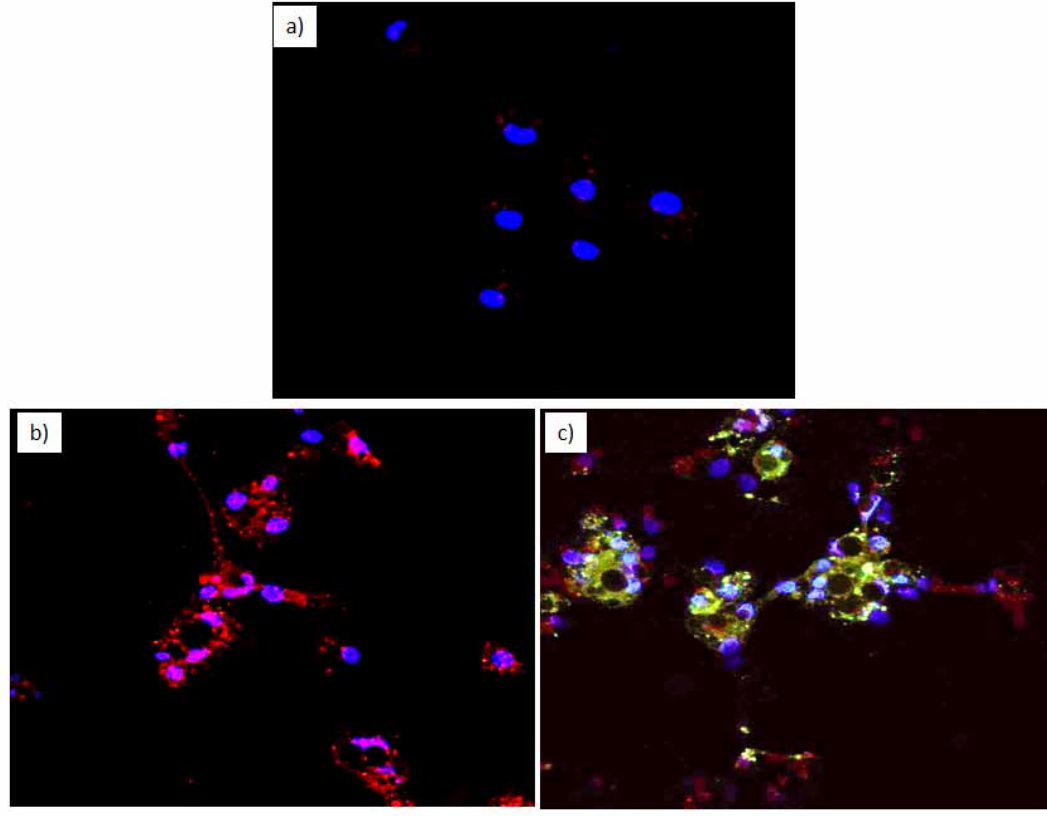
Figure 4. Annexin V/PI Apoptotic Analysis of BM-DCs following LPS Treatment and vvIBDV Infection at 3, 6, 12 and 24 hpi. Note: a) Control BM-DCs; b) LPS-treated BM-DCs and c) vvIBDV-infected BM-DCs. (i) Lower left box indicate percentage of live BM-DCs. (ii) Lower right and (iii) upper right indicate percentage of early and late apoptosis of BM-DCs, respectively. (iv) Upper left box indicate percentage of necrotic BM-DCs. The data shown are representative of three independent experiments. Statistical differences of (i) Live BM-DCs: Control^A, 3H LPS^B, 6H LPS^B, 12H LPS^B, 24H LPS^B, 3H vvIBDV^A, 6H vvIBDV^B, 12H vvIBDV^C, 24H vvIBDV^C; (ii) Early Apoptosis: Control^A, 3H LPS^A, 6H LPS^A, 12H LPS^A, 24H LPS^A, 3H vvIBDV^A, 6H vvIBDV^A, 12H vvIBDV^A, 24H vvIBDV^B; (iii) Late Apoptosis: Control^A, 3H LPS^{AB}, 6H LPS^{BC}, 12H LPS^{ABC}, 24H LPS^C, 3H vvIBDV^{AB}, 6H vvIBDV^{AB}, 12H vvIBDV^C,

24H vvIBDV^D; (iv) Necrosis: Control^A, 3H LPS^B, 6H LPS^C, 12H LPS^{AD}, 24H LPS^{CD}, 3H vvIBDV^B, 6H vvIBDV^A, 12H vvIBDV^{ACD}, 24H vvIBDV^{CD}. *^{ABCD} Values with different superscripts differ significantly at $P < 0.05$.

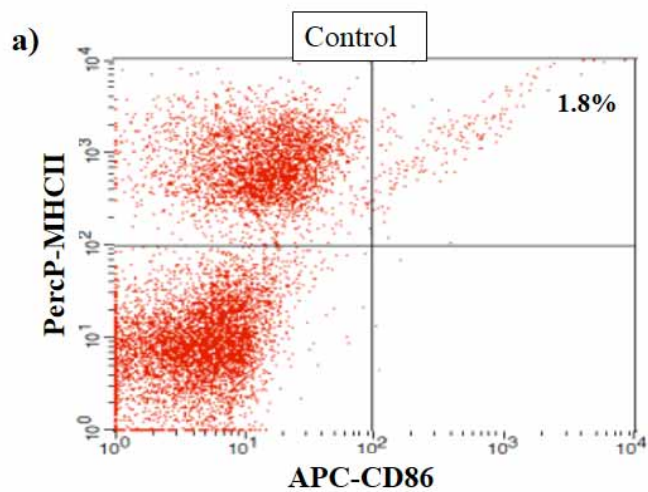
Figure 5. Quantification of mRNA levels of selected immune-related genes in control BM-DCs, LPS-treated BM-DCs and IBDV-infected BM-DCs at 3, 6, 12 and 24 hpi. Note: Data are expressed as fold change in cytokine mRNA levels compared with those in control BM-DCs. The data shown are representative of three independent experiments. ^{ABCDEF} Values with different superscripts differ significantly at $P < 0.05$ compared to control. Inactivated vvIBDV-infected BM-DCs failed to induce the expressions of IFN- γ , IL-12 α and TLR3

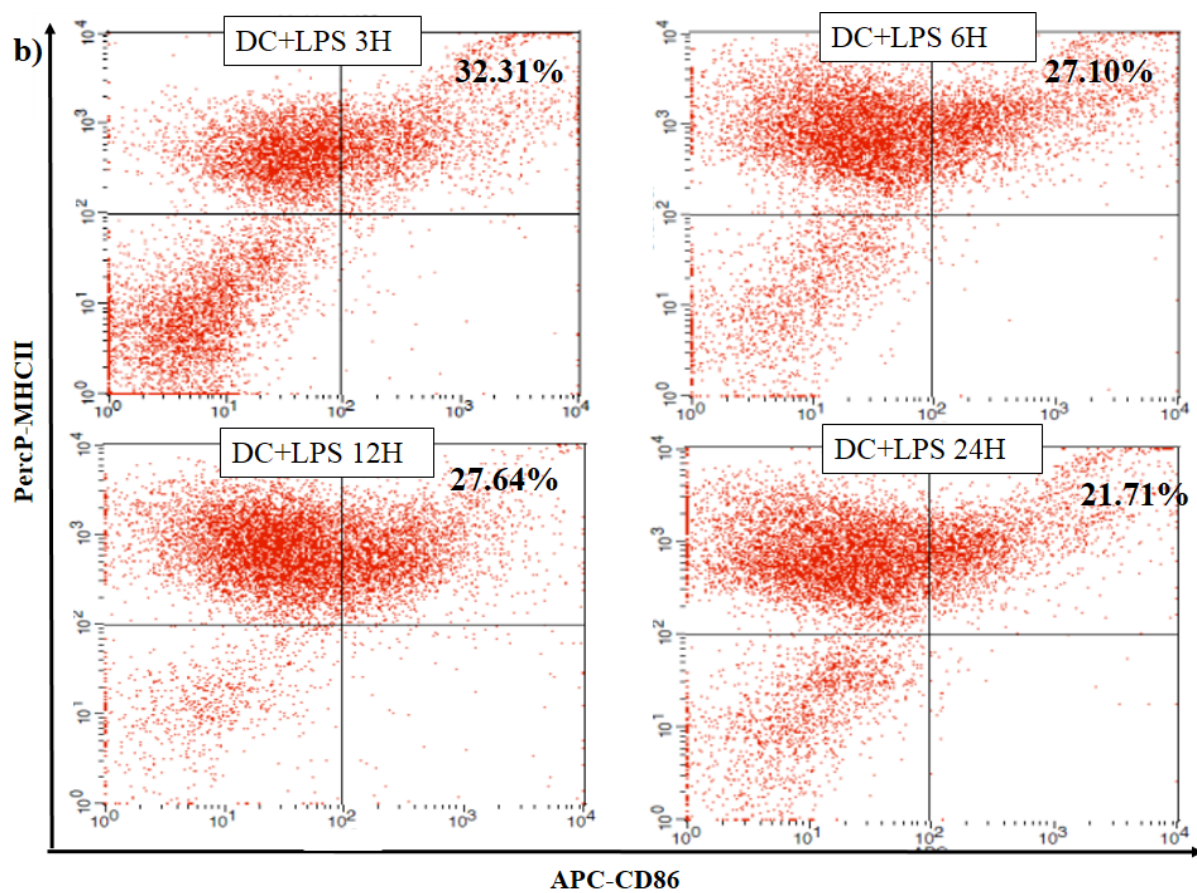


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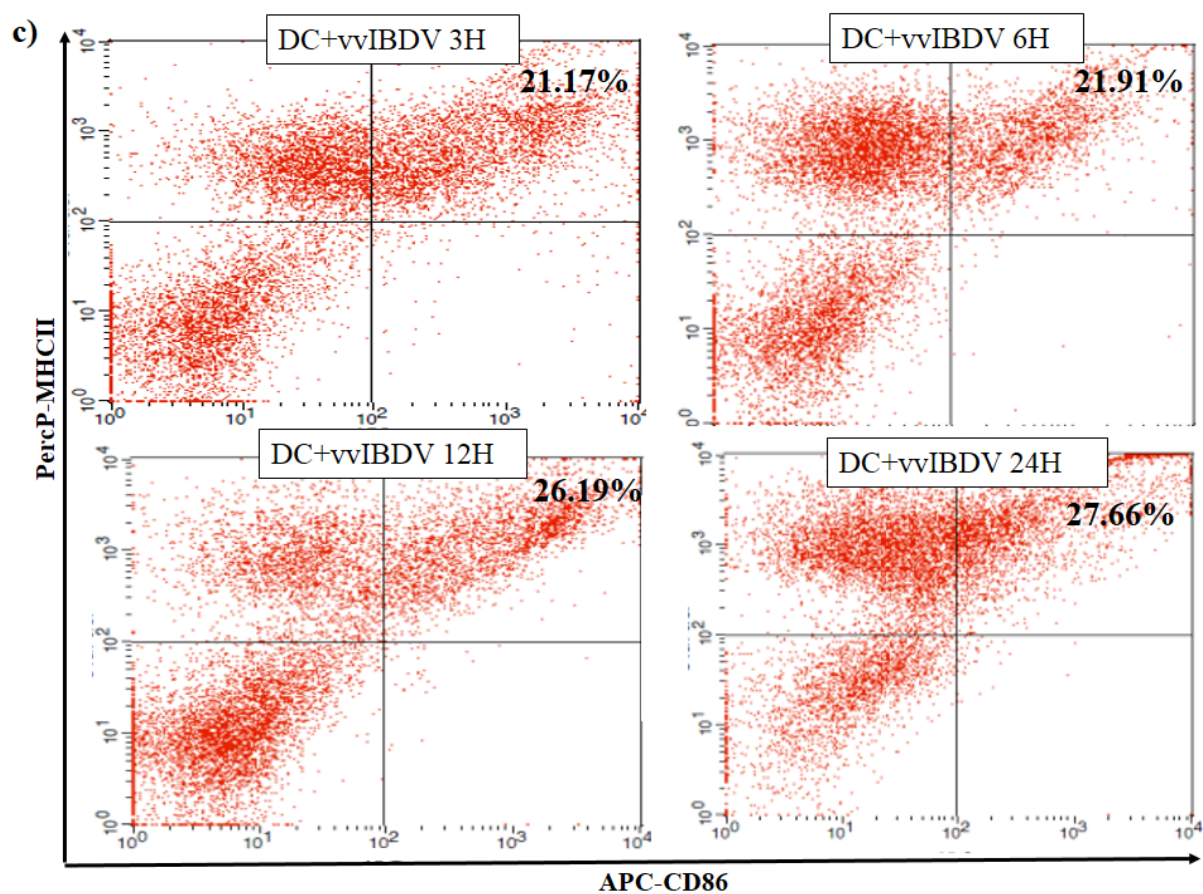


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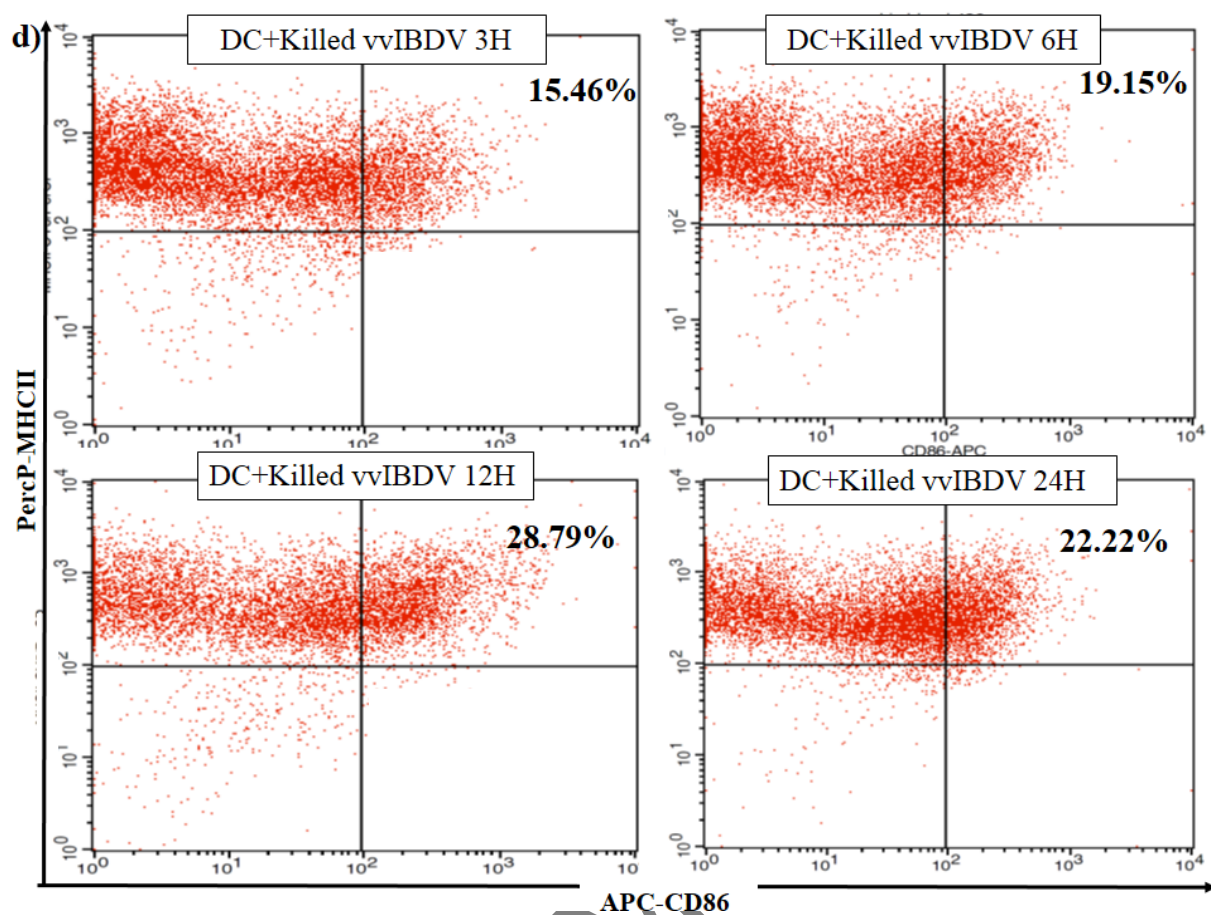




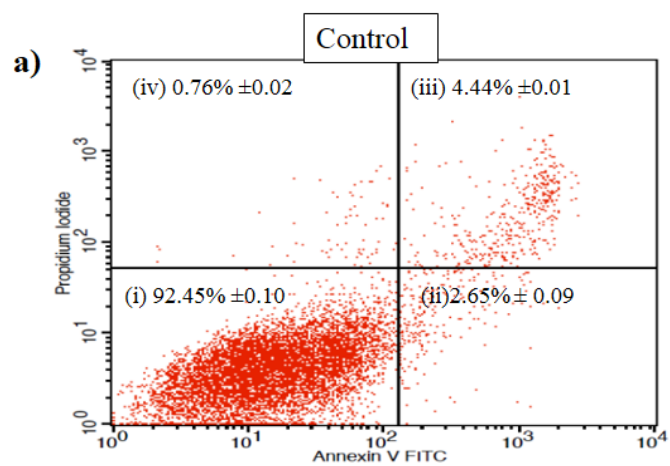
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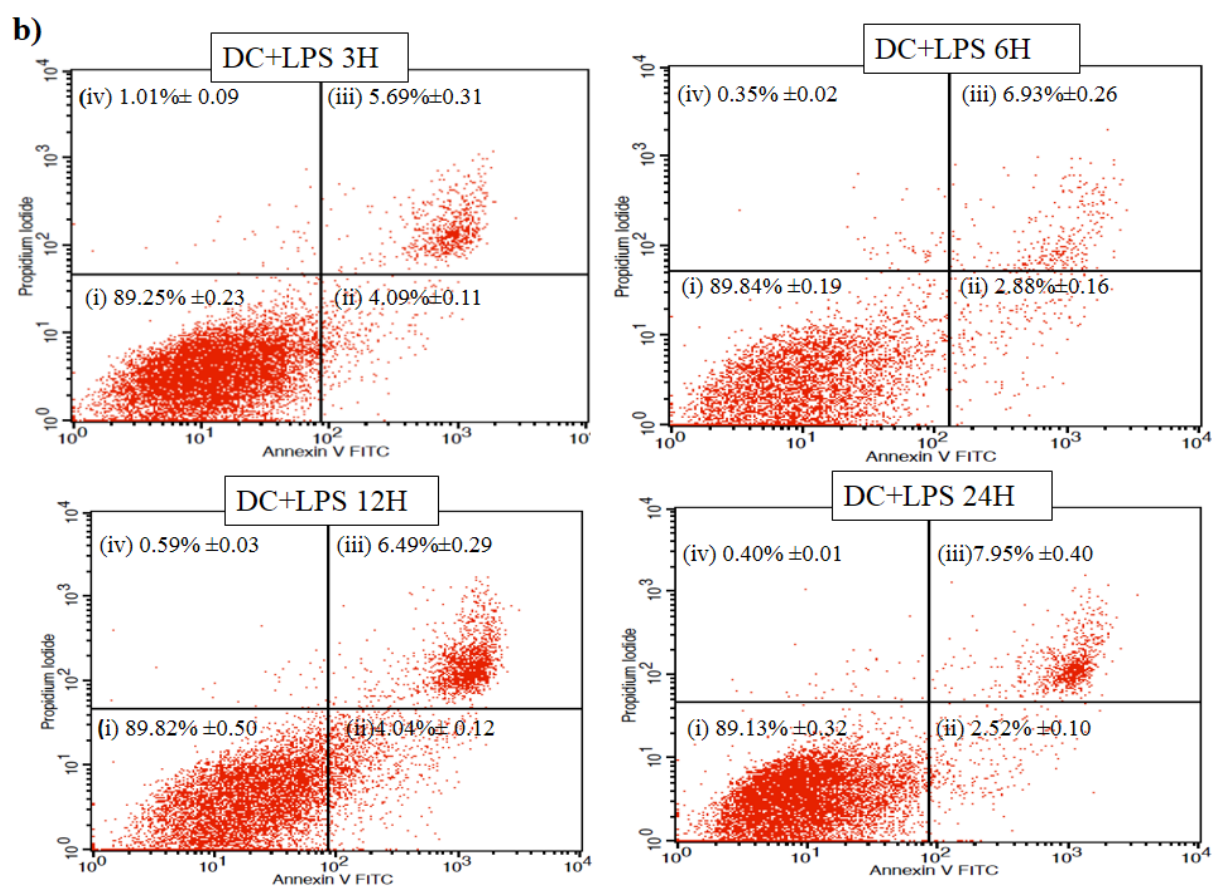
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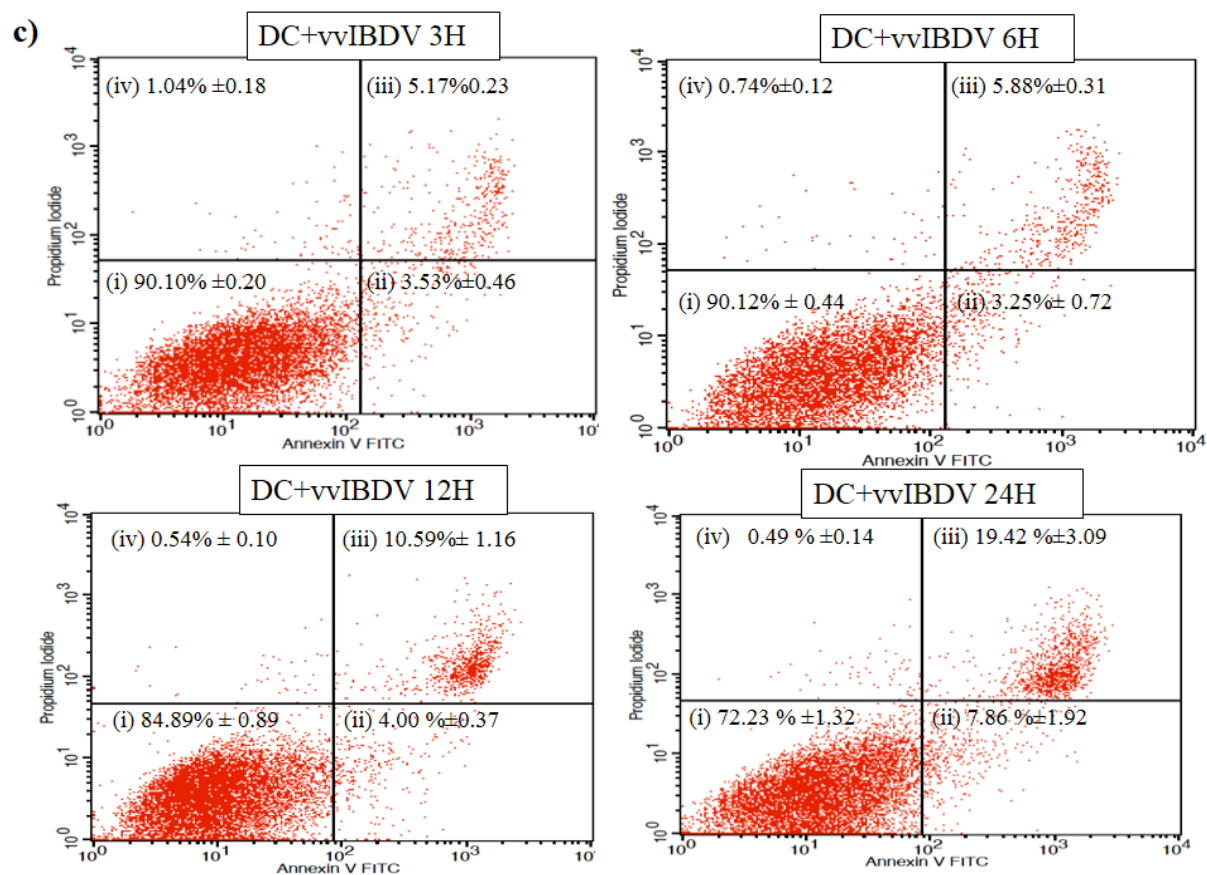
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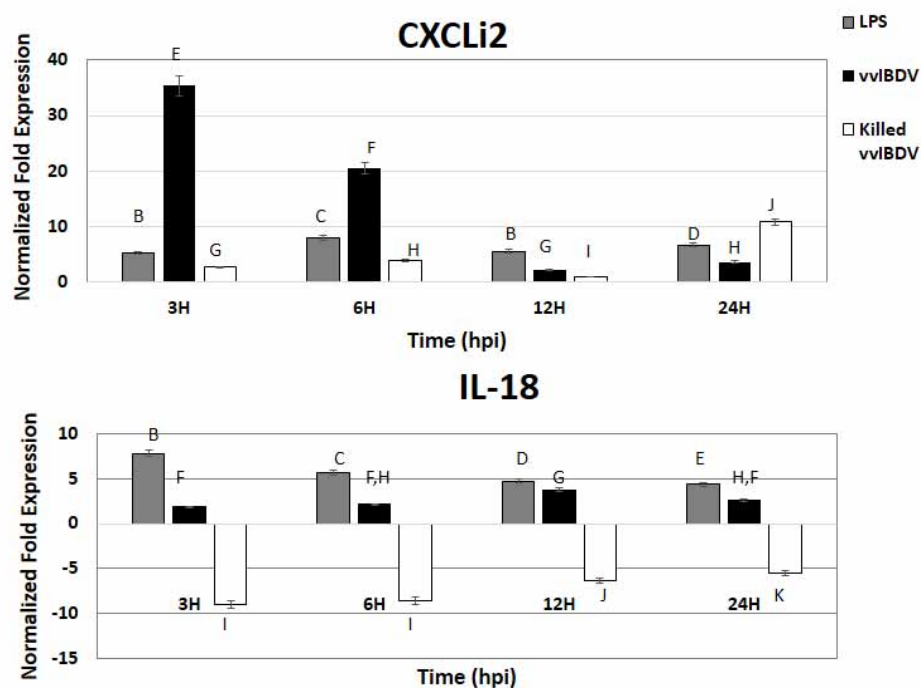


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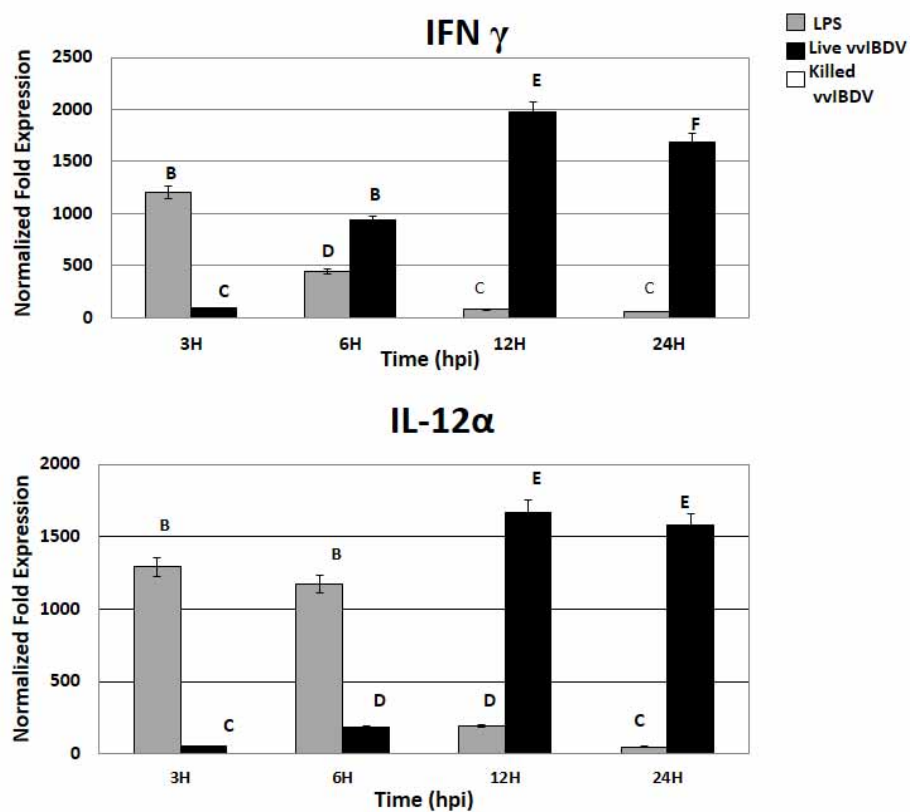


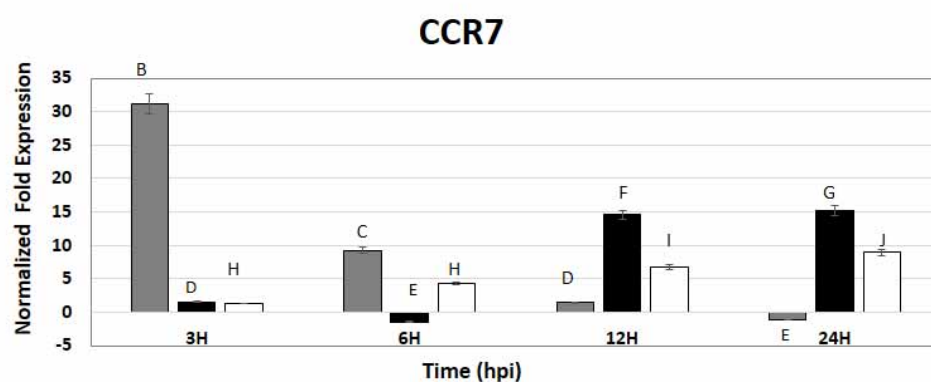
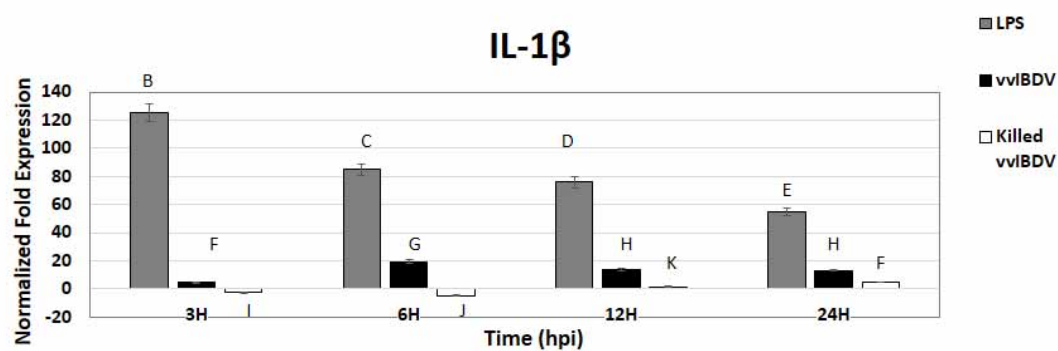
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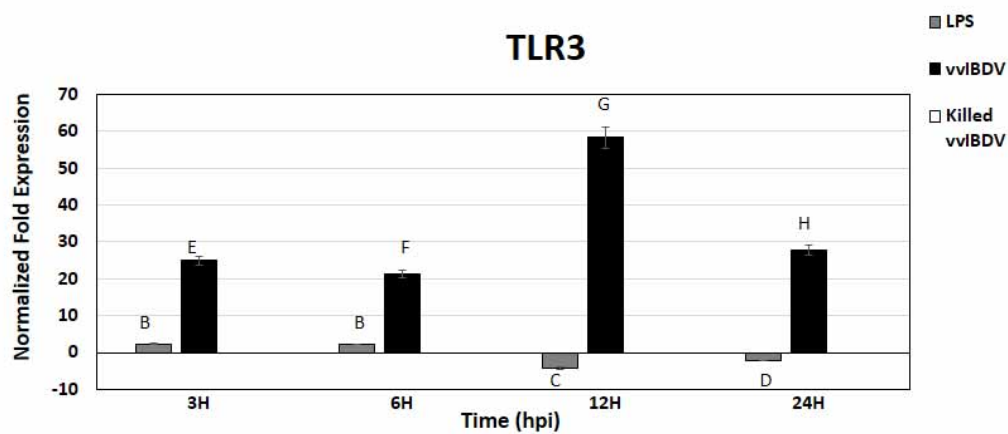


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